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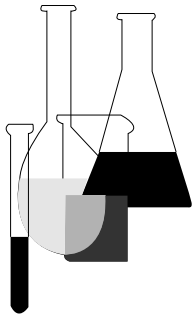
Ecological Effects Test Guidelines

OPPTS 850.4225 Seedling Emergence, Tier II



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 850.4225 Seedling emergence, tier II.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline 40 CFR 797.2750 Seed Germination/Root Elongation Toxicity Test and 797.2800 Early Seedling Growth Toxicity Test and OPP 123–1 Seed Germination/Seedling Emergence and Vegetative Vigor (TierII) (Pesticide Assessment Guidelines, Subdivision J—Hazard Evaluation; Nontarget Plants) EPA report 540/09-82-020, 1982.

(b) **Tier II—Seedling emergence dose response testing.** This guideline should be used in conjunction with OPPTS guideline 850.4000, Background—Nontarget plant testing, which provides general information and overall guidance for the nontarget plants test guidelines.

(1) **Objective.** (i) The terrestrial nontarget plant phytotoxicity tests are laboratory tests that evaluate the acute toxicity of pesticides to 10 plant species. These studies evaluate the effects of multiple dosage levels on plant growth, using less than the maximum label rate with dosages in a geometric progression of no more than two-fold, and with subtoxic (<EC50) and nontoxic (no observable effect level, NOEL) concentrations. The typical end-use product is used, and the test takes a minimum of 14 days. Results are reported as EC25, EC50 and NOEC (or EC05) values in pounds active ingredient (AI) per acre. Parameters measured include percent emergence, number of emerged plants, seedling height, seedling dry weight, root length, and root dry weight (if a root active compound), and observed phytotoxicity. The results are used to establish acute toxicity levels, compare with measured or estimated environmental concentrations, and to indicate if further testing at a higher tier is necessary.

(ii) Nontarget plant phytotoxicity data are used to assess whether the potential hazard of chemicals to nontarget plants—terrestrial, semiaquatic, or aquatic. Nontarget plants include plants outside the area of intended application (which would include food and cover vegetation for animals, food, fiber, fuel, and ornamental plants for man, and endangered and threatened plants). Phytotoxicity data are occasionally requested in order to assess the potential hazard of certain pesticides to plants within the treatment area (target area testing). Terrestrial plants serve as food and shelter for fish and wildlife, help control erosion, and serve as air pollution filters. Shoreline vegetation buffers reduce siltation and provide an environment in which aquatic invertebrates can contribute to the food supply of fish, reptiles, and amphibians. Vegetation adjacent to streams serves to regulate water temperature which in turn contributes to improved aquatic life. Decaying vegetation provides nutrients essential for the aquatic

food chain. Hedgerows, woodlots, and other similar nontarget areas provide food and cover to mammalian and avian species.

(iii) Nontarget plant phytotoxicity data are routinely used to conduct ecological risk assessments for the registration and reregistration of pesticides. These data are also useful in our assessment of potential hazards to endangered/threatened plant species listed by the Department of Interior, Fish and Wildlife Service. Tier II (Definitive Studies) tests are initiated following a determination that a greater than 25 percent adverse effect occurred in the Tier I (Initial Screening Study) seedling emergence study for one or more plant species. If less than a 25 percent detrimental effect or response to all tested species is noted in the seedling emergence study, no higher tier testing is ordinarily required. Under FIFRA, if the pesticide is a known phytotoxicant, terrestrial plant testing begins with Tier II. Unique agency concerns arising from 6(a)(2) incidents (adverse effects reporting), Special Review issues, pesticide contamination cases, published literature, or other public sources may result in requests for additional tests.

(c) **Test methods**—(1) **Test facility/location/test conditions.** Dose response seedling emergence tests can be conducted in the greenhouse or in small field plots. Report soil type and texture, soil K_d and K_{oc} values and pH. Environmental conditions during the test should be recorded daily—light intensity, air temperature, humidity, photoperiods, thermoperiods, watering schedules and methods (rainfall if field test), and pest conditions.

(2) **Test substance.** Refer to 40 CFR part 160 for test substance requirements. Use of TEP instead of TGAI is preferred for all nontarget plant phytotoxicity tests, using the TEP with the highest percent AI and/or the one most commonly used.

(3) **Controls/solvents/additives and other pesticide treatments.** Pesticide treatments other than the test pesticide should be avoided. Mechanical, cultural, and biological pest control methods are suggested. If solvents or other pesticides are used in the test, the registrant must show that the solvent/pesticide is not toxic to the test species and that no synergistic or antagonistic interactions with the test pesticide exists (additional test data). To demonstrate solvent or adjuvant activity, a separate set of control plants (set aside for this purpose at the beginning of the experiment) can be treated with the solvent/pesticide using the highest dosage. A negative control is still required. If the solvent/pesticide controls and the negative control are contaminated with the test chemical, the study should most likely be repeated. If the solvent control is contaminated with the test chemical and the negative control is not (and visa versa), the study may not be invalid if zero percent toxicity occurred in the negative control and at the lowest dose tested. Within a given study, all test organisms including the controls should be from the same source. To prevent bias, a system of random assignment of the test plants to test and control groups

is required. If adjuvants are recommended on a TEP label, a representative adjuvant within each class (ionic, anionic, nonionic, etc.) must be used in the test.

(4) **Equipment.** (i) All equipment used in conducting the test, including equipment used to prepare and administer the test substance, and equipment to maintain and record environmental conditions, should be of such design and capacity that tests involving this equipment can be conducted in a reliable and scientific manner. Equipment should be inspected, cleaned, and maintained regularly, and be properly calibrated.

(ii) The application equipment used in testing products in small field plot studies should be designed to simulate conventional farm equipment using the basic components of commercial application equipment in the design of the small-plot equipment. For example, nozzle types, sizes, and arrangements on small plot sprayers can be identical to those used by growers on commercial ground sprayers. Specific details as to descriptions of equipment design, adjustment, and operation should be provided in test reports.

(5) **Dosages.** (i) At least five dosages should be tested.

(ii) The dosages should include a subtoxic (<EC50) and a nontoxic concentration.

(iii) The dosages should be of geometric progressions of no more than fourfold. An example of a twofold series: 0.1, 0.2, 0.4, 0.8, and 1.6 kg/ha. A twofold progression is preferred, however, threefold and fourfold progressions are acceptable with the proviso that the test be extended for the most sensitive test species if the no-effect level (or EC05) has not been achieved. The test must define the response curve so that accurate EC50, EC25, and NOEC (or EC05) values can be obtained. The lack of a no-effect level is not critical as long as the slope is adequate for calculation of valid EC50, EC25, and EC05 values from the most sensitive endpoints (e.g. dry shoot weight, dry root weight, shoot height). The lowest test level and the NOEC should not be greater than the EC25 value.

(iv) Dosages should be reported in units of AI or acid equivalent as appropriate. Rates may be expressed as units of ingredient per unit of land area to be treated, units of concentration (such as parts per million), units per flow rate, or units of ingredient per unit volume applied to obtain a specified degree of foliage coverage (such as ‘‘to runoff’’). Under FIFRA, if a pesticide is applied more than once within a year or growing season, each rate and the interval between applications should be indicated. If products are applied in a tank mixture or are applied serially, rates and intervals, as appropriate, should be reported with identification and formulation for each product.

(v) If conducting tests under TSCA, the test chemical is applied daily with each watering for the duration of the study.

(6) **Plant test species.** (i) At least three replicates, each with 10 plants, should be tested per dose level. Larger populations and more replicates of certain plants with low germination may be needed to increase the statistical significance of the test.

(ii) Healthy plants must be used. Pesticide treated seeds should be avoided. The Agency should be consulted prior to test initiation if seed treatments other than steam, a weak hypochlorite solution (recommended by Environment Canada), captan, or thiram are used. Captan and thiram seed treatments are the only approved pesticide seed treatments (noninteractive with most other pesticides). Steam sterilization of soil and seeds is the recommended procedure for killing pathogens, fungi, and insects on seeds and in soil media. Some methods used to remove seed treatments include rinsing with a weak methanol solution, detergents, or hypochlorite solution. When unapproved pesticide seed treatments are used in a study, it is the responsibility of the laboratory conducting the test to show that no synergistic or antagonistic interactions occur between or among the various pesticides in the test.

(iii) Ten plant species must be tested. (A) The following plant species and groups are recommended:

(*1*) Dicotyledoneae: Six species of at least four families, one species of which is soybean (*Glycine max*) and a second of which is a root crop.

(*2*) Monocotyledoneae: Four species of at least two families, one species of which is corn (*Zea mays*).

(B) Of the 10 test plants, 3 must be tested: corn (*Zea mays*), soybean (*Glycine max*), and a root crop such as carrot (*Ducas carrotta*), onion (*Allium cepa*), beet (*Beta vulgaris*), or sugarbeet (*Beta vulgaris*). The other seven test species might include: tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativa*), lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), oat (*Avena sativa*), and perennial ryegrass (*Lolium perenne*). Substitution of other test species (other crops, weeds controlled, native plants, perennials, woody species) when species sensitivity to the test compound is known ahead of time is encouraged. Endangered or threatened species as determined by the Endangered Species Act of 1973 (Public Law 93–205) may not be used without permission from the Fish and Wildlife Service.

(7) **Support media and pesticide dosing method.** Seeds may be germinated in pots using a sterilized standardized soil that consists primarily of sandy loam, loamy sand, loamy clay, or clay loam soil that contains up to 3 percent organic matter. The use of 100 percent acid washed sand or hydroponic methods (glass beads, rockwool, etc.) are not recommended.

Test methods and protocols for hydroponic tests should be submitted to the Agency for review prior to test initiation. Depending on the intended application method, the pesticide is either thoroughly mixed into the soil (incorporated to label or TSCA specified soil depth) or applied to the soil surface using a properly calibrated sprayer.

(8) **Test containers.** Test containers should be nonporous so that the test material is not absorbed. Do not use clay or peat containers. Containers should be thoroughly cleaned prior to use. A dichromate solution should not be used to clean containers.

(9) **Test parameters.** (i) Carbon dioxide level should be maintained at 350 ± 50 ppm.

(ii) Relative humidity should approach 70 ± 5 percent during light periods and 90 percent during dark periods.

(iii) Irradiation measured at 400 to 700 nm at 1 m from the source, at 350 ± 50 $\mu E/m^2$ sec.

(iv) Photoperiods of 16 h light and 8 h darkness.

(v) Day/night temperatures at $25\ °C/20\ °C \pm 3\ °C$.

(vi) Half-strength modified Hoagland nutrient solution may be used as nutrient medium.

(10) **Watering methods.** Bottom watering of test containers is preferred in order to prevent washing the chemical through the soil during watering. The laboratory should assess the potential for leaching of the pesticide based on solubility and K_d value before attempting the top watering method.

(11) **Plant density.** While the number of plants per pot is left to the discretion of the laboratory conducting the test, avoid overcrowding. As an example, one to two corn, soybean, tomato, cucumber, or sugarbeet plants per 6 inch container; three rape or pea plants per 6 inch container; and a maximum of six onion, wheat, or other small grain per 6 inch container. The test conditions should approximate those optimal conditions for the species and varieties tested (cool and warm climate crops should be tested separately).

(12) **Test duration/end-points.** (i) The test plants are observed upon emergence from the soil and seedling emergence is recorded as the number of emerged seedlings. Shoot heights and visual phytotoxicity ratings are recorded on days 7 and 14 (or more frequently), as compared to the control plants. On the final day of the study, shoot heights and shoot dry weights are also recorded, in addition to visual phytotoxicity ratings and total number of dead plants per dosage level per replicate. Root dry weight and length measurements are optional, only required if the pesticide inhibits

root formation (based on mode-of-action information, incident reports, literature). Individual test species responses to cultural conditions, mode-of-action of the pesticide and speed of uptake of the pesticide by the test plants are factors that affect the interval between pesticide application and final observations for adverse effects. The study length should be extended as dictated by these factors. All dead plants are to be measured, weighed and observed and included in the statistical analysis.

(ii) Observations should include all variations, either inhibitory or stimulatory, between the treated and the untreated organisms. Such variations may be phytotoxic symptoms (chlorosis, necrosis, and wilting), formative (leaf and stem deformation) effects, and/or growth and development rates. Observations should include the stage of development and dates when adverse results occurred and subsided or recovered. Any lack of effects by the pesticide should also be reported. Include actual counts, weights, and other measurements for each plant, replicate and variable. Uniform scoring procedures should be used to evaluate the observable toxic responses. Such data should include the actual values used to determine any percentages of effects. Raw data (chromatographs, field reports, and analysis data) may also be included to substantiate the basic data that are required.

(13) **Minimum seed germination standards.** The following minimum acceptable USDA seed germination (control) standards for vegetable crops (as described in the Federal Seed Act Regulation, 7 CFR parts 201-202) and other available standards for agronomic crops will be used: Field corn (85%), pop corn (75%), sweet corn (75%), carrot (55%), onion (70%), tomato (75%), field-garden bean (70%), pea (80%), pepper (55%), beet (65%), buckwheat (60%), cabbage (75%), lettuce (55%), mustard—all types (75%), soybean (75%), sugarbeet (55%), small grains—wheat, oats, barley, rice (80%), ryegrass (75%), vetch (75%), alfalfa—clover (70%), rape (75%). Refer to regulation for additional vegetable crops.

(d) **Reporting requirements**—(1) **General.** Refer to 40 CFR 160.185, subpart J, for reporting requirements. Report should include name of laboratory or test location, personnel information, test substance information, test procedures, materials, methods, results, and analysis of data in tabular summary form. Statistical methods must be described. Report the actual dates of the studies including dates of initiation (planting, transplanting, and cultural practices), applications, observations, and harvest. Information on mode-of-action (biochemical) and resultant plant effects should be included. Either the metric system or the U.S. standard measures may be used in test reports. The U.S. standard measures may be used to preclude extensive conversion to the metric system. The two systems should not be mixed (e.g., grams per square feet). The English language is to be used in all test reports. English translations must be provided with foreign language reports.

(2) **Test report.** The test report must include the following information:

(i) The number of seeds sown and the number emerged per dosage level for each replicate compared to controls.

(ii) Descriptions of the appearance and the growth and development of the emergent plants, indicating any abnormalities and expressions of phytotoxicity.

(iii) Tabulation of the results indicating measured differences in emergence, height, dry weight, phytotoxicity, and total number of dead plants for treated plants compared to control plants (individual counts/measurements may be combined for each replicate for statistical purposes).

(iv) Electronic transfer of test data on discs is encouraged to reduce review time.

(3) **Statistics.** Statistical analysis is required to evaluate the responses when test results such as efficacy, phytotoxicity, or yield indicate adverse effects on crops and other nontarget test organisms, The statistical analysis should consist of:

(i) The tabulation of data for the most sensitive endpoint (plant height, plant dry weight, visual phytotoxicity, etc.) for each plant species tested at each treatment level for each test.

(ii) The determination of 25 and 50 percent detrimental effect levels (EC25, EC50) and the 95 percent confidence limits, where possible, for each (Probit Analysis, Bruce/Versteeg).

(iii) The estimated NOEC (or EC05) and LOEC (Williams Test).

(e) **Special test requirements.** In addition to the data required in this guidance, data from other tests may be required by the Agency for making judgments regarding safety to nontarget plants such as additional test species, life-cycle tests, and monitoring studies. Such data will be required where there are special concerns identified in the literature, 6(a)(2) or incident action, a unique use pattern, or a unique chemical property.

(f) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Boutin, C. et al. Proposed Guideline For Registration Of Chemical Pesticides: Nontarget plant testing and evaluation. Tech. Rpt. Series No. 145, Canadian Wildlife Service, Environment Canada, pg. 1–91 (1993).

(2) Bruce, R.D. and D.J. Versteeg. A Statistical Procedure For Modeling Continuous Toxicity Data. *Environmental Toxicology and Chemistry* 11:1485-1494 (1992).

(3) Gulley, D.D. et al. Toxstat. Release 3.0. University of Wyoming, Laramie, WY (1989).

(4) EPA. Nontarget Plants: Seed Germination/

eedlingEmergence—Tiers I and II. EPA 540/9–86–132 (1986).

(5) EPA. Nontarget Plants: Target Area Testing. EPA 540/9–86–130 (1986).

(6) Stephan, C.E. Methods for calculating an LC50. In F.L. Mayer and J.L. Hamelink, eds., Aquatic Toxicology and Hazard Evaluation, STP 634, American Society for Testing and Materials, Philadelphia, PA, pp. 65-84 (1977).

(7) Truelove, B., (ed). *Research Methods in Weed Science*. Southern Weed Science Society, Auburn Printing Inc., Auburn, AL 36830 (1977).